

# Endogenous DHP-sensitive $\text{Ca}^{2+}$ channels in *Pleurodeles* oocytes

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## Abstract

The double electrode voltage-clamp technique was used to study voltage-dependent  $\text{Ca}^{2+}$  channels in *Pleurodeles* oocytes. From a holding potential of  $-80$  mV, Ba-current ( $I_{\text{Ba}}$ ) (recorded in Cl-free solution,  $\text{Ba}^{2+} = 40$  mM) activated at  $-36.7 \pm 4$  mV, peaked at  $-11.6 \pm 4$  mV and reversed at  $55 \pm 7$  mV ( $n = 24$ ). This current activated slowly (rise time was  $0.98 \pm 0.2$  s;  $n = 14$  at  $-10$  mV) and was not inactivated. Cadmium ( $\text{Cd}^{2+}$ ,  $500 \mu\text{M}$ ) completely inhibited  $I_{\text{Ba}}$ . The effect of  $\text{Cd}^{2+}$  was dose-dependent ( $\text{EC}_{50} = 37 \pm 5 \mu\text{M}$ ;  $n = 5$ ). Moreover,  $I_{\text{Ba}}$  was insensitive to  $\omega$ -conotoxin ( $10 \mu\text{M}$ ) but interestingly this  $I_{\text{Ba}}$  displayed dihydropyridine (DHP) sensitivity. Bay K 8644 ( $5 \mu\text{M}$ ), a DHP activator, increased the peak current amplitude in a dose-dependent manner ( $\text{EC}_{50} = 5.9 \pm 0.6 \mu\text{M}$ ;  $n = 10$ ) and shifted the threshold and the maximum of current/voltage relationship towards negative potentials by  $-10$  mV. Nifedipine ( $5 \mu\text{M}$ ), a DHP antagonist, decreased  $I_{\text{Ba}}$  by 80% at HP of  $-80$  mV ( $\text{EC}_{50} = 1.2 \pm 0.2 \mu\text{M}$ ;  $n = 6$ ). We concluded that *Pleurodeles* oocytes possess High-Voltage Activated  $\text{Ca}^{2+}$  channels with properties similar to L-type  $\text{Ca}^{2+}$  channels.

**Key words:** *Pleurodeles* oocytes; Dihydropyridine;  $\text{Ca}^{2+}$  channel; Voltage-clamp

## 1. Introduction

A variety of oocytes produce action potentials when their membrane is depolarized. In invertebrates oocytes, the action potentials result from the activation of both  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  currents while in vertebrate oocytes, purely calcium or sodium dependent spikes are observed [1,2,3].  $\text{Ca}^{2+}$  channel multiplicity has been extensively demonstrated in a variety of cell types [4,5] including oocytes and eggs [1,6]. In ascidian eggs, two different  $\text{Ca}^{2+}$  channels have been identified: one, the 'high-voltage-activated' (HVA), so-called L-type  $\text{Ca}^{2+}$  channel (DHP-sensitive); the other one is a 'low-voltage-activated' (LVA), so-called T-type  $\text{Ca}^{2+}$  channel [7]. In the immature oocytes from the starfish *Mediaster*, both HVA and LVA  $\text{Ca}^{2+}$  channels coexist [8]. However, only HVA  $\text{Ca}^{2+}$  channels have been recorded in *Xenopus* oocytes [9]. To date, there have been no electrophysiological studies on  $\text{Ca}^{2+}$  channels in *Pleurodeles* immature oocytes, while a rise of intracellular  $\text{Ca}^{2+}$  activity has been observed during progesterone induced maturation [10]. With  $\text{Ba}^{2+}$  ions as charge carriers, we found HVA  $\text{Ca}^{2+}$  channel currents with very slower kinetics of activation and inactivation. Interestingly, these currents are sensitive to dihydropyridines, agonist (Bay K 8644) and antagonist (nifedipine).

## 2. Materials and methods

### 2.1. Oocyte preparation

All experiments were performed during the breeding season (September to May) when *Pleurodeles* oocytes resumed meiosis with progester-

one treatment. Oocytes were dissected away from ovary of female *Pleurodeles waltlii* (urodele amphibian, from Laboratoire de Biologie du Développement, USTL, France) anaesthetized with 1% MS 222 (tricaine methanesulfonate; Sandoz) and prepared as reported previously [11]. Stage VI oocytes (diameter of 1.6 mm) were selected for electrophysiological measurements. Defolliculated oocytes could be maintained for 2–6 days at  $19^{\circ}\text{C}$  in control solution containing (ND 96; in mM): NaCl 96, KCl 2,  $\text{CaCl}_2$  2 and HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] 10, pH 7.2 with NaOH and supplemented with  $50 \mu\text{g/ml}$  gentamicin. Incubation medium was renewed daily.

### 2.2. Electrophysiological measurements

Electrophysiological measurements were performed using the standard two microelectrode voltage-clamp technique with TEV-200 amplifier (Dagan Instruments, Minneapolis, MN). Stimulation of the preparation, data acquisition and analysis were performed using the pCLAMP software (ver. 5.5, Axon Instrument, Burlingame, CA). Oocytes were placed in a recording chamber ( $300 \mu\text{l}$ ) and impaled with 3 M CsCl-filled microelectrodes ( $0.2\text{--}1 \text{ M}\Omega$ ).

Drugs were applied externally by addition to the superfusate (gravity driven superfusion). We used a Cl free solution (CaMS; in mM): NaOH 96, KOH 2, CaOH 2 and HEPES 10, pH 7.2 (methane sulfonic acid) in order to block the Cl currents. The main part of the outward K current was inhibited by using Cs instead of K in the recording electrodes and 4-aminopyridine ( $10 \text{ mM}$ ) was added in the CaMS and in the BaMS solutions. No fast Na current was detected in these oocytes. However, all extracellular Na was replaced by tetraethylammonium and tetrodotoxin ( $10 \mu\text{M}$ ) was added. Thus, to record  $\text{Ca}^{2+}$  channel activity, oocytes were tested in the high barium methane sulfonate solution (BaMS, in mM): TEAOH 50, CsOH 2, BaOH 40 and HEPES 10, pH adjusted to 7.2 with methane sulfonic acid.

### 2.3. Drugs

Cadmium, 4-aminopyridine, tetraethylammonium and tetrodotoxin were purchased from Sigma (St. Louis, MO, USA) and were dissolved in water. The DHP agonist Bay K 8644 and antagonist nifedipine (Calbiochem, France) were dissolved in 50% ethanol to make concentrated stock solutions ( $10 \text{ mM}$ ) and stored at  $-20^{\circ}\text{C}$ .  $\omega$ -Conotoxin was purchased from Calbiochem (France) and was dissolved in water to make stock solution ( $10 \text{ mM}$ ). All final concentrations were obtained by appropriate dilution in BaMS solution.

### 2.4. Analysis

Ba-current was measured as the difference between the maximal inward current amplitude and the zero current level. Most data are expressed as the means (S.E.M.) ( $n$  = number of experiments).

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### 3. Results

The mean membrane potential from immature oocytes was  $-63 \pm 10$  mV ( $n = 10$ ) and the membrane capacitance was  $0.19 \pm 0.04$   $\mu$ F ( $n = 16$ ).

#### 3.1. Electrophysiological properties of the $I_{Ba}$ -current

In a normal physiological bathing medium (ND 96), depolarization to +20 mV from a holding potential of -80 mV elicited an outward current (Fig. 1A<sub>1</sub>). The perfusion of a Cl-free solution (CaMS;  $Ca^{2+} = 2$  mM) reduced this outward current (Fig. 1A<sub>2</sub>). This result suggests that a portion of outward current is carried by Cl ions. The perfusion of the high  $Ba^{2+}$  solution (BaMS;  $Ba^{2+} = 40$  mM) blocked completely the remaining outward current and elicited an inward  $Ba$ -current (Fig. 1A<sub>3</sub>). Moreover, *Pleurodeles* oocytes developed a  $Ca^{2+}$  activated  $K^+$  current which activated about -40 mV. Fig. 1B shows the  $Ca^{2+}$  activated  $K^+$  current elicited from a holding potential of -80 mV to -10 mV during exposure to high  $Ca^{2+}$  solution (CaMS;  $Ca^{2+} = 20$  mM). An inward current was elicited when  $Ca^{2+}$  (20 mM) was replaced by  $Ba^{2+}$  (20 mM) (Fig. 1B). However, even in BaMS solution, a small outward current remained at above +30 mV.

This paper is based on recordings  $Ca^{2+}$  channel activity in *Pleurodeles* oocytes. Fig. 1C shows typical waves of  $I_{Ba}$  evoked for various depolarizations between -40 to +30 mV from a holding potential of -80 mV. The peak  $I_{Ba}$  magnitudes were normalized for variation in oocytes size using oocytes membrane capacitance. The mean of  $I_{Ba}$  density records during a test pulse to -10 mV from a holding potential of -80 mV was  $0.86 \pm 0.21$   $\mu$ A/ $\mu$ F ( $n = 24$ ).  $I_{Ba}$  density/voltage relationship is shown in Fig. 1D. For test pulses of a 2 s duration,  $I_{Ba}$  activated at  $-36.7 \pm 4$  mV ( $n = 24$ ), peaked at  $-11.6 \pm 4$  mV ( $n = 24$ ) and reversed at  $55 \pm 7$  mV ( $n = 24$ ). However, in few oocytes no inward  $Ba$ -current could be recorded except in the presence of the DHP agonist (Bay K 8644) (data not shown). Thus, only a HVA  $Ca^{2+}$  channel is evident in the *Pleurodeles* oocytes. Using a 2 or 6 s pulses, we were unable to observe any inactivation of  $I_{Ba}$  (data not shown). Furthermore,  $I_{Ba}$  showed extremely slow inactivation requiring 3 min pulse duration (Fig. 1E).

Further characterization of these  $Ca^{2+}$  channels has been focussed on the effect of various classical  $Ca^{2+}$  channel inhibitors or activators used to differentiate between the different types of HVA  $Ca^{2+}$  channels.

#### 3.2. Effect of $Ca^{2+}$ on $I_{Ba}$

Among the many potent inorganic  $Ca^{2+}$  channel blockers,  $Cd^{2+}$  is of particular interest in a number of preparations. Fig. 2A shows effect of different concentrations of  $Cd^{2+}$  on HVA  $Ba$ -current activated from -80 mV to -10 mV. The reduction of  $I_{Ba}$  by  $Cd^{2+}$  was dose-

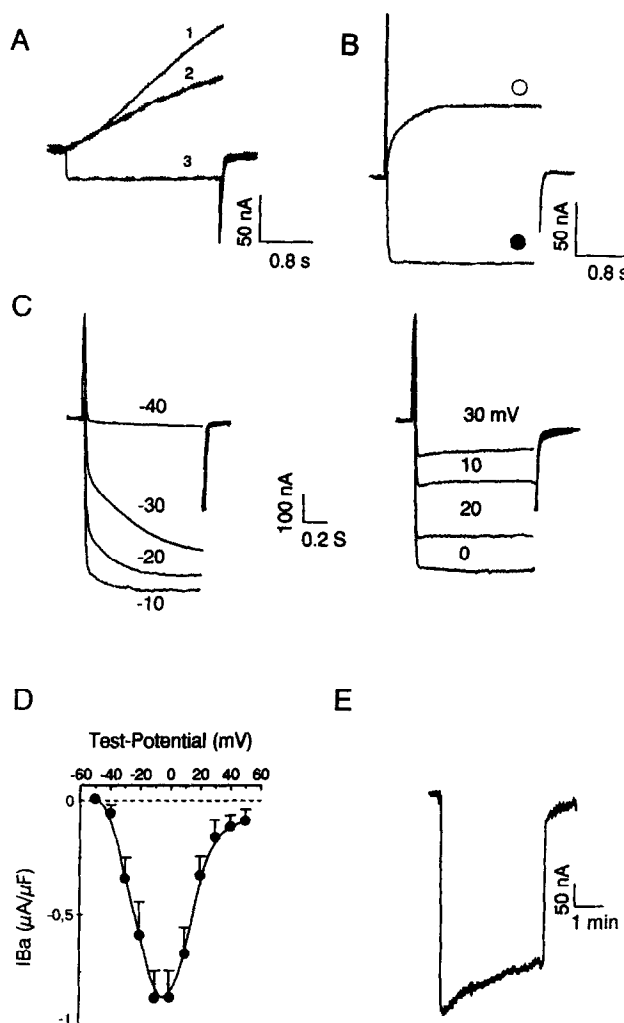


Fig. 1. (A–E) Electrophysiological properties of 'high-voltage activated' calcium channel currents (using  $Ba^{2+}$  as charge carrier) in *Pleurodeles* oocytes. (A) Typical traces showing an outward current recorded: (1) in normal physiological medium (ND 96); (2) in the free Cl solution (CaMS;  $Ca^{2+} = 2$  mM); and (3) the recovery of  $I_{Ba}$  when superfused with high BaMS solution (0 Cl, 40 mM  $Ba^{2+}$ ). The holding potential ( $V_h$ ) was stepped from -80 mV to +20 mV for 2 s. (B) Outward current elicited from  $V_h$  of -80 mV to -10 mV (○) in the high CaMS solution (0 Cl, 20 mM  $Ca^{2+}$ ). The substitution of external  $Ca^{2+}$  (20 mM) by  $Ba^{2+}$  (20 mM) elicited an inward  $I_{Ba}$  (●). (C) Typical waveforms of  $I_{Ba}$ . From  $V_h$  of -80 mV,  $I_{Ba}$  was evoked by test pulses to depolarization between -40 mV and -10 mV (left panel) and between 0 mV and +30 mV (right panel) presented in 10 mV increment. (D) Current/voltage relationship of the peak current normalized by the membrane capacitance. Mean  $I_{Ba}$  density ( $\mu$ A/ $\mu$ F) are plotted as a function of depolarization  $\pm$  S.E.M. ( $n = 24$ ). (E) Kinetics of activation and inactivation of  $I_{Ba}$ . The waveforms of  $I_{Ba}$  evoked at -10 mV from  $V_h$  of -80 mV for 3 min duration.

dependent with an  $EC_{50}$  of  $37 \pm 5$   $\mu$ M ( $n = 5$ ; Fig. 2B). A complete block of  $I_{Ba}$  was obtained in the presence of 500  $\mu$ M  $Cd^{2+}$  (Fig. 2A,B,C). Fig. 2C shows peak  $I/V$  relationship determined in the absence and the presence of 500  $\mu$ M  $Cd^{2+}$ .

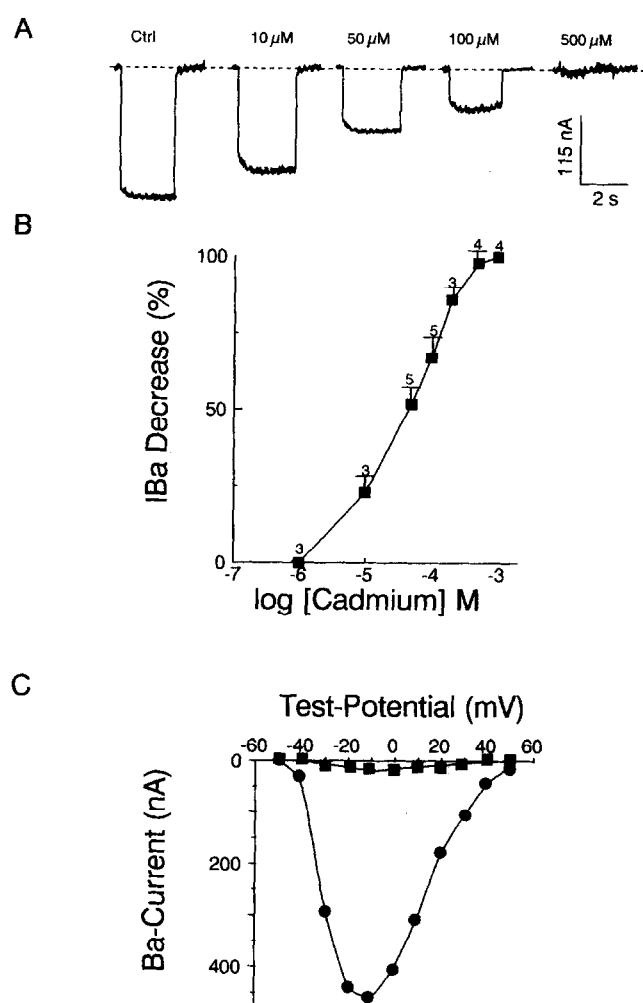


Fig. 2. (A–C) Effects of various concentrations of Cd<sup>2+</sup> on  $I_{Ba}$ . (A) Effect of Cd<sup>2+</sup> (10, 50, 100, 500 μM) on  $I_{Ba}$ . The holding potential was stepped from –80 mV to –10 mV for 2 s (Ctrl = control). (B) Cumulative dose/response relationship of the inhibition of  $I_{Ba}$  by Cd<sup>2+</sup>. Each point is expressed as mean ± S.E.M.,  $n$  = number of oocytes. The dose of 50% inhibition is  $37 \pm 5$  μM ( $n = 5$ ). (C) Current/voltage relationship of  $I_{Ba}$  (●) before and (■) after perfusion of Cd<sup>2+</sup> (500 μM).  $V_h$  was –80 mV, test pulses 2 s in duration and 10 mV in increment were applied every 40 s.

### 3.3. Effect of $\omega$ -conotoxin ( $\omega$ -cgTx) and dihydropyridines (DHP) on $I_{Ba}$

In many tissues, the entry of Ca<sup>2+</sup> ions can be modulated by diverse classes of organic compounds that have proved their efficiency as ligands to purify and characterize Ca<sup>2+</sup> channels.

Sensitivity to  $\omega$ -cgTx and 1–4 dihydropyridines has been used to identify different subtypes of Ca<sup>2+</sup> channels.  $\omega$ -cgTx has no effect on L-type Ca<sup>2+</sup> channels in smooth, cardiac and skeletal muscle but reduces the N-type Ca<sup>2+</sup> channels [12]. When  $\omega$ -cgTx was applied in concentrations with a saturating block in other preparations (10 μM), in *Pleurodeles* oocytes, no effect on  $I_{Ba}$  was observed ( $n = 12$ , data not shown).

The primary site of action of 1–4 dihydropyridines is

thought to be L-type Ca<sup>2+</sup> channels [13]. Bay K 8644, a dihydropyridine agonist, has been shown to promote Ca<sup>2+</sup> current [14,15]. External application of Bay K 8644 (5 μM) increased the peak inward current at test potential from –80 mV to –20 mV (Fig. 3A). The effect of Bay K 8644 on  $I_{Ba}$  was dose-dependent with an EC<sub>50</sub> of  $5.9 \pm 0.6$  μM ( $n = 10$ ) (Fig. 3B). The threshold and the maximum of the  $I/V$  relationship (Fig. 3C) shifted about –10 mV in the negative direction consistent with the shift in voltage dependence of activation seen in heart [16,17], neurones [18] and skeletal cells [19].

The effect of the DHP antagonist nifedipine on  $I_{Ba}$  is shown in Fig. 3. The peak inward current was significantly reduced by nifedipine (5 μM) at test potential –20 mV (Fig. 3D). The effect of nifedipine on  $I_{Ba}$  was dose-dependent with an EC<sub>50</sub> of  $1.2 \pm 0.2$  μM ( $n = 6$ ) (Fig. 3E). By contrast to the effect of Bay K 8644, no leftward shift in the  $I/V$  curve was obtained with nifedipine (Fig. 3F).

## 4. Discussion

The main result of our study is the existence of HVA Ca<sup>2+</sup> channel DHP-sensitive in the *Pleurodeles* oocytes. These HVA Ca<sup>2+</sup> channels, like others found in various preparations [5,13] can be distinguished by the activation voltage ( $\approx -30$  mV) and their sensitivity to the inorganic blocker Cd<sup>2+</sup> (EC<sub>50</sub> =  $37 \pm 5$  μM, Fig. 2). Moreover,  $I_{Ba}$  reported here was modulated by DHP agonist and antagonist. Bay K 8644, a DHP activator, enhances the peak  $I_{Ba}$  and induces a left shift by about –10 to –15 mV of both threshold and the maximum of the  $I/V$  curve. Nifedipine, a DHP antagonist, reduces  $I_{Ba}$  by  $\approx 80\%$  at HP of –80 mV and induces no leftward shift in the  $I/V$  curve (Fig. 3D–F). Taken together, these results agree well with those obtained on cardiac [16,17] and skeletal L Ca<sup>2+</sup> channels [19,20]. However, some discrepancies can be observed between our results and those derived from cardiac and skeletal cells: (i) the DHP concentrations used to obtain the half maximum effect in our experiments are 100–1000-fold higher than those reported in cardiac ( $\approx 10^{-8}$  M for both nifedipine and Bay K 8644 [14,21] and in skeletal cells ( $\approx 10^{-9}$  M) [19]. However, it should be noted that these (EC<sub>50</sub>) from cardiac and skeletal cells were obtained in the presence of the normal external calcium while in our experiments we use a 40 mM external Ba<sup>2+</sup> concentration; (ii) in skeletal muscle, Bay K 8644 shifted only the threshold of  $I/V$  curve [19] while in our study both the threshold and the peak of the  $I/V$  curve are shifted in the presence of Bay K 8644.

So, what types of channel mediates  $I_{Ba}$  in *Pleurodeles* oocytes? In terms of the voltage-dependent properties and sensitivity to DHP (agonist and antagonist), this channel resembles the L-type Ca<sup>2+</sup> channel reported in

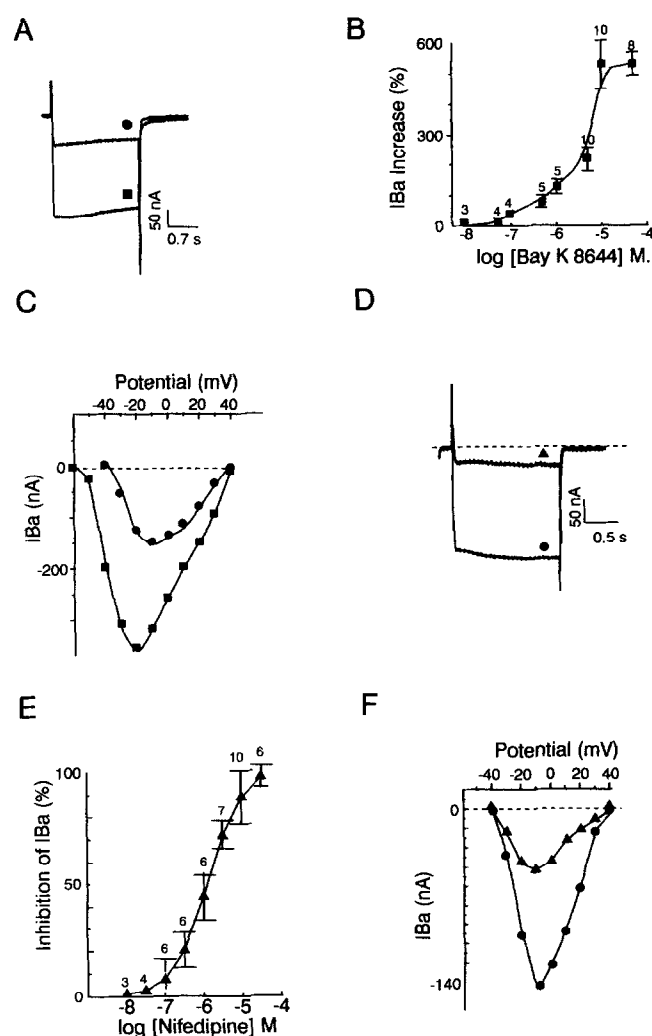


Fig. 3. (A–F) Regulation of  $I_{Ba}$  by DHPs agonist (Bay K 8644) and antagonist (nifedipine). (A) Individual Ba-current traces evoked by a depolarizing pulse from  $-80$  mV to  $-20$  mV; (●)  $I_{Ba}$  recorded in the control solution and (■) maximal increase of  $I_{Ba}$  induced by perfusion with the same solution containing Bay K 8644 ( $5 \mu\text{M}$ ). (B) Cumulative dose/response relationship of increase  $I_{Ba}$  by Bay K 8644. The dose of 50% increase is  $5.9 \pm 0.6 \mu\text{M}$  ( $n = 10$ ). Each point is expressed as mean  $\pm$  S.E.M.,  $n$  = number of oocytes. (C) Current/voltage relationship traced from  $V_h -80$  mV (●) in absence and (■) in presence of Bay K 8644 ( $5 \mu\text{M}$ ). The threshold and the maximum of  $I_{Ba}$  were shifted by about  $-10$  mV towards negative potentials. (D) Individual Ba-current traces recorded from  $-80$  mV to  $-20$  mV, (●)  $I_{Ba}$  control, (▲)  $I_{Ba}$  in the presence of nifedipine ( $5 \mu\text{M}$ ). (E) Cumulative dose/response relationship of decrease  $I_{Ba}$  by nifedipine. The dose of 50% decrease is  $1.2 \pm 0.2 \mu\text{M}$  ( $n = 6$ ). Each point is expressed as mean  $\pm$  S.E.M.,  $n$  = number of oocytes. (F) Current/voltage relationship traced from  $V_h -80$  mV (●) in absence and (▲) in presence of nifedipine ( $5 \mu\text{M}$ ).

various tissues: cardiac [17,22], neuronal [18,23] and skeletal [20,24]. In terms of the activation kinetic (time to peak,  $0.98 \pm 0.2$  s at  $-10$  mV),  $I_{Ba}$  closely resembles the slow activating DHP-sensitive  $\text{Ca}^{2+}$  channel reported in skeletal muscle [24,25]. In terms of inactivation kinetics (non-inactivating  $I_{Ba}$ ),  $I_{Ba}$  is similar to the fast rising

non-inactivating DHP  $\text{Ca}^{2+}$  channel reported in dysgenic skeletal muscle [26].

Recently, molecular biological studies have revealed that the HVA  $\text{Ca}^{2+}$  channel is a protein complex containing  $\alpha_1$ ,  $\alpha_2/\delta$ ,  $\beta$  and  $\gamma$  subunits [27,28]. Recent studies have revealed a diversity among L-type  $\text{Ca}^{2+}$  channels arising from: (i) multiples genes encoding the pore forming  $\alpha_1$ -subunit; and (ii) various genes (at least 4) encoding the auxiliary  $\beta$ -subunit. While it is already known that the  $\alpha_1$ -subunit plays a major role in determining the activation kinetics [27,28], the auxiliary subunits, in particular  $\beta$ -subunit plays a major role in determining both the kinetics and voltage dependence of the inactivation process. In L-cells, stable expression of skeletal muscle  $\alpha_1$  alone was sufficient to generate a slowly activating and inactivating  $\text{Ca}^{2+}$  channel [29,30]. The coexpression of skeletal muscle  $\beta$  with skeletal muscle  $\alpha_1$  markedly accelerate both the activation and inactivation kinetics [29,30]. As reported here  $I_{Ba}$  was activated slowly and did not inactivate may indicate that the L-type  $\text{Ca}^{2+}$  channels in *Pleurodeles* oocytes contain  $\alpha_1$ -subunit (DHP sensitive) but a few and/or no  $\beta$ -subunit.

Our study argues an heterogeneity in  $\text{Ca}^{2+}$  channel types between amphibian oocytes and more generally between vertebrate and invertebrate oocytes: (i) T and L  $\text{Ca}^{2+}$  channels in ascidian eggs and starfish oocytes [7,8]; (ii) HVA  $\text{Ca}^{2+}$  channels different from the four subtypes known in *Xenopus* oocytes [9,31,32] and (iii) a DHP-sensitive HVA  $\text{Ca}^{2+}$  channel subtype in the *Pleurodeles* oocytes. These  $\text{Ca}^{2+}$  channels (DHP-sensitive) in the *Pleurodeles* oocytes might have a physiological involvement in the regulation of free internal Ca concentration during meiosis, fertilization and development.

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